The Role of Curcumin and Diclofenac Sodium in Modulating Anti-Arthritic and Anti-Angiogenic Activities: An In-vitro Evaluation

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Abstract

Osteoarthritis is a degenerative bone disorder characterized by cartilage destruction and bone remodelling. Multiple risk factors like age, sex, trauma, obesity and genetic predisposing factors are linked with osteoarthritis. Inflammation and angiogenesis are the main characteristic features of osteoarthritic pathophysiology. The present research was aimed to evaluate the *in vitro* anti-arthritic and anti angiogenic activity of curcumin, diclofenac sodium and their combination. Anti arthritic activity was evaluated by protein denaturation using bovine serum albumin. Denaturation was induced by incubating the drugs with bovine serum albumin under experimental conditions and compared with the control. The protein denaturation was quantified by measuring their absorbance at 660nm. Percentage of Inhibition and IC50 were calculated. Anti-angiogenic effect was evaluated using Chick Chorion Allantoic Membrane (CAM) assay. Drugs were loaded on to the agar gel and aspectically inserted into CAM. Angiogenesis was evaluated by counting the number of blood vessels under microscope. The length, diameter of primary, secondary and tertiary blood vessel and CAM area of different groups were measured by using Image J software. The results demonstrates that diclofenac sodium and curcumin when given in combination induced more effective anti-arthritic and anti-angiogenic effect than the individual drugs. The results of this study highlighted the synergistic potency of diclofenac sodium and curcumin combination in attenuating arthritis and angiogenesis which can serve as a novel therapeutic strategy in treatment of osteoarthritis

Introduction

Osteoarthritis (OA) is a degenerative disorder characterized by cartilage degeneration, inflammation, remodelling of adjacent bone leading to intense joint pain accompanied by limitation in mobility and reduced quality of life. OA is also known as ‘wear and tear’ arthritis. Genetics, sex, trauma, age and obesity are the major risk factors associated with OA. Articular cartilage provides surface for the movement of synovial joints, is often affected by OA. It is composed of hyaline cartilage and proteoglycans and type II collagen rich Extra Cellular Matrix (ECM). Articular cartilage is usually avascular in nature, but found to undergo extensive vascularisation during OA due to hypoxic conditions induced by multiple factors including oxidative stress and mechanical strain. Being a chronic inflammatory disorder, several biochemical changes and simultaneous destruction of cartilage leads to synovitis, characterized by alterated chondrocyte function, enhanced angiogenesis and decreased bone turnover. Recruitment of activated macrophages and lymphocytes into the synovial capsule leads to release of several pro inflammatory and pro catabolic mediators alterating the vascularity of synovial membrane. These activated macrophages may directly or indirectly release angiogenic factors like VEGF and TNF-α. Concurrent increase in metabolic demand in synovial cells results in hypoxia, which in turn increases the recruitment of macrophages adding to the effect of angiogenesis. Hypoxic tissues induce Hypoxia inducible factor –α (HIF-α) stimulating synovial cells and macrophages to secrete Vascular Endothelial Growth Factor (VEGF) leading to angiogenesis.

Several pharmacological and non-pharmacological interventions presently in practice are narrowed due to their associated adverse effects. Pain alleviation and improvement of quality of life remains the primodial goal in the treatment of OA. Conventionally, several NSAIDs like acetaminophen, diclofenac and Ibuprofen are employed to relieve pain. These drugs act by inhibiting Cyclo-Oxygenase enzyme thereby decreasing the production of inflammatory mediators. Due to their non specificity in inhibiting COX enzyme, these drugs are often associated with several adverse effects which are of major concern.

Curcumin, a yellow colored pigment obtained from the rhizomes of *Curcuma longa Linn* belonging to the family Zingiberaceae, composed of Curcumin (70-76%), demethoxycurcumin (16%) and bisdemethoxycurcumin (8%) as the major constituents. It is an extensively used Indian spice and is known for its antibacterial, anti inflammatory, anti fungal, anti viral, anti tumor, anti spasmodic effects and anti retro-viral properties. The present study focuses on the evaluation of *in-vitro* anti arthritic and anti-angiogenic activity of curcumin compared to the standard drug Diclofenac sodium in isolation and in combination.

Material and methods

1. Drugs and chemicals:

Curcumin and Diclofenac sodium were purchased from Hychem Laboratories, Hyderabad. *In-vitro* anti-arthritic and anti-angiogenic activity were estimated by protein denaturation method and chorionic allantoin membrane (CAM) assay.

2. Estimation of in-vitro anti arthritic activity by protein denaturation method:

Protein denaturation by using bovine serum albumin was done by using the method of Pavitra TK et.al.[2,3] The reaction mixture (0.5 ml) consisted of 0.45 ml bovine serum albumin (5% aqueous solution) and 0.05 ml of Test extracts (100 and 250 mcg/ml of final volume). pH was adjusted at 6.3 using a small amount of 1 N HCl. The samples were incubated at 37˚C for 20 min and then heated at 57˚C for 3 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm for control test 0.05 ml distilled water was used instead of extracts while product control test lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows.

$$\text{Percent inhibition} = \frac{100 - (\text{O.D. of test} - \text{O.D. of product control}) \times 100}{\text{O D of control}}$$

The control represents 100% protein denaturation. The results were compared with Diclofenac sodium treated sample.

3. Chorioallantoic Membrane (CAM) assay:

Collection of fertilized chicken eggs:

The fertilized eggs were obtained from Venkateshwara central poultry breeding farm Hyderabad, Telangana, India.

Assay:

For the Chorioallantoic membrane CAM assay, fertilized chick embryos were pre-incubated for 8 days at 37.5℃ in 85% humidity. A hole was drilled over the air sac at the end of the eggs and an avascular zone was identified in the CAM. A 1 cm×1 cm window in the shell was sectioned to expose the CAM. Eggs were divided into 4 groups each containing 10 eggs. Group A eggs were control (Fig 1), Group B received Diclofenac sodium (0.2%) (Fig 2), Group C received Curcumin (0.2%) (Fig 3), Group D received both Diclofenac sodium (0.2%) and Curcumin (0.2%) (Fig 4) on the CAM surface. Windows were sealed with clear tape and eggs were incubated for 48 h. Blood vessels were viewed and photographed with Nikon digital camera. The anti-angiogenic effects of the drugs on CAMs were quantified by counting the number of blood vessel branch points [4].

Results

1. In-vitro anti-arthritic activity:

The drug concentration for 50% inhibition was determined by plotting percent inhibition with respect to control against concentration (µg/ml) taken in the range 25-250 µg/ml. The results (Mean±S.D) values are summarized in Fig 1.

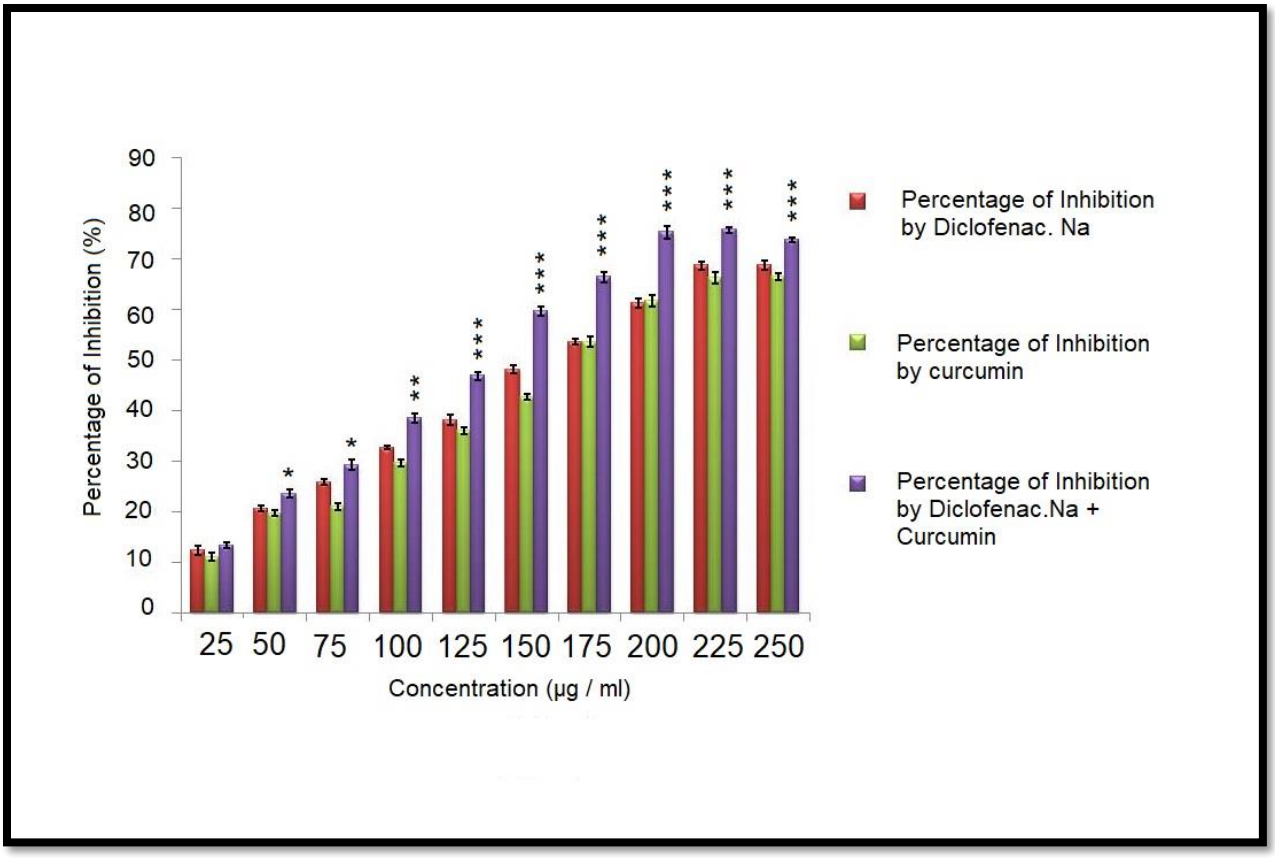

Fig 1: Effect of diclofenac sodium, curcumin and their combination at a concentration range of 25-250µg/ml on protein denaturation using bovine serum albumin. Data was represented as mean ± S.D and was analysed by one way ANOVA followed by significance indices are inverted in the figure intentionally due to space constraints. The data was represented as mean±S.D. Data was analysed by one way ANOVA followed by posthoc Bonferomi test and p value > 0.05 was considered to be significant. * p>0.005, ** p > 0.01, *** p > 0.001.The IC50 values for Diclofenac sodium, Curcumin and their combination was found to be 171.48µg/ml, 163.76 µg/ml and 136.56µg/ml respectively

From the above results it is evident that the combination Curcumin and Diclofenac sodium possess more anti inflammatory action when compared to individual drugs.

2. CAM assay:

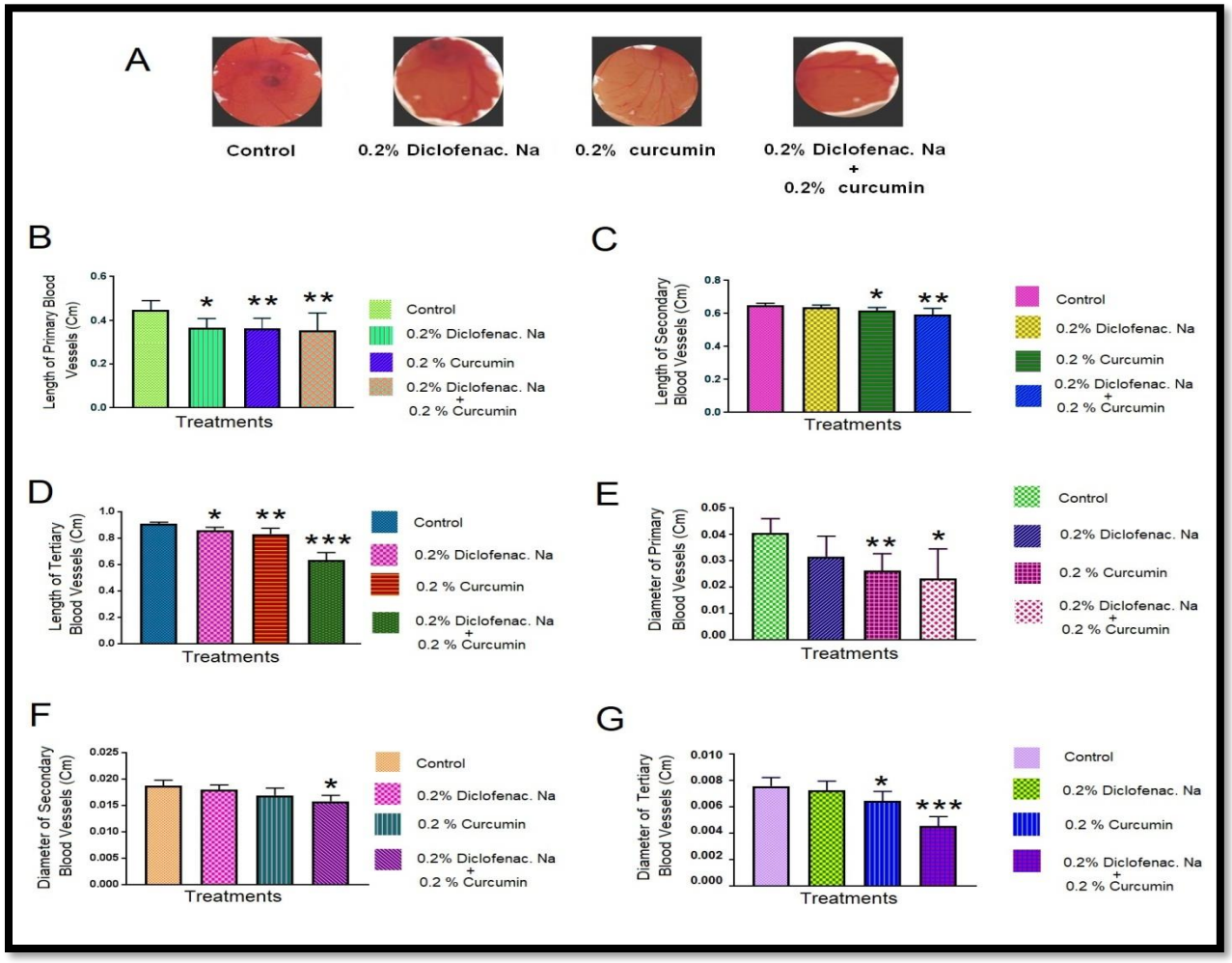

Figure 2: Effect of diclofenac sodium, curcumin and their combination at a concentration of 0.2% (w/v) on angiogenesis using CAM assay where A is a pictorial representation of exposed CAM from various treatment groups. B is the effect of various treatments on the length of primary blood vessels expressed in cm. C is the effect of various treatments on the length of secondary blood vessels. D is the effect of various treatments on the length of tertiary blood vessels. E is the effect of various treatments on the diameter of primary blood vessels. F is the effect of various treatments on the diameter of secondary blood vessels. G is the effect of various treatments on the diameter of tertiary blood vessels. The data was represented as mean±S.D. Data was analysed by one way ANOVA followed by posthoc Bonferomi test and p value > 0.05 was considered to be significant. * p>0.005, ** p > 0.01, *** p > 0.001.

Effect of various treatments on the length of primary blood vessels:

The length of the primary blood vessels of group D (0.3551±0.0791) was significantly reduced when compared to group B (0.3664±0.0428) and Group C (0.3639±0.0428) eggs which were treated with Diclofenac sodium and curcumin respectively. Group A (0.4481±0.0425) was considered as control with no treatment. (Fig 2, B)

Effect of various treatments on the length of secondary blood vessels:

The length of secondary blood vessels of Group D (0.5921±0.0390) has significantly decreased when compared to Group C (0.6165±0.0197). The length secondary blood vessels in Group B (0.6365±0.0149) were also decreased when compared to Group A (0.6519±0.0106) but the decrease in length was not significant (Fig 2, C).

Effect of various treatments on the length of tertiary blood vessels:

The decrease in the length of tertiary blood vessels is highest in Group D (0.6337±0.0580) significantly and then in Group C (0.8311±0.0444). The length was also decreased in Group B (0.8590±0.0239) when compared to control Group A (0.9086±0.0135) non significantly (Fig 2, D).

Effect of various treatments on the diameter of primary blood vessels:
The diameter of primary blood vessels was significantly decreased in Group D (0.0232±0.0112) when compared to Group C (0.0263±0.0064) and Group B (0.0316±0.0077). The treated groups were compared with the control Group A (0.0404±0.0055) (Fig 2, E).

Effect of various treatments on the diameter of secondary blood vessels:
The diameter of secondary blood vessels was found to decrease in Group D (0.0158±0.0011) significantly when compared to Group C (0.0169±0.0014) and Group B (0.0180±0.0009). All the treated groups were compared with the control Group A (0.0187±0.0011) (Fig 2, F).

Effect of various treatments on the diameter of tertiary blood vessels:
The diameter of tertiary blood vessels was found to be decreased in Group D (0.0046±0.0007) compared to Group C (0.0065±0.0007) and Group B (0.0073±0.0007). All the treated groups were compared to control Group A (0.0076±0.0007) (Fig 2, G).

Discussion

Osteoarthritis is characterized by the enhanced denaturation of type II Collagen in articular cartilage [5]. Protein denaturation a common feature of both osteoarthritic cartilage and senescent cartilage, Nevertheless, observed predominantly in osteoarthritic degeneration contrary to physiological cartilaginous aging by virtue of increased levels of degradation enzymes. Post translational modifications induced in the proteins by modifying the hydrophobic, electrostatic and hydrogen bonds as well as disulfide bridges in the protein tertiary structure result in protein degeneration [6]. Henceforth therapeutic strategies that attenuate protein degeneration serve as a viable option for the management of osteoarthritis. In the light of these indications, the present study focused on the evaluation of curcumin, diclofenac sodium and their combination against their ability to inhibit protein denaturation using in vitro model. The study indicated that both the drugs possess anti arthritic activity but exhibited a heightened response when used in combination in comparison with per se controls as evidenced by the IC50 values of percentage of inhibition.

Angiogenesis is a hallmark feature of inflammatory disorders. Although considered to be an integral part of healing response, angiogenesis contributes widely to the pathological progression of various inflammatory disorders. A critical balance between the pro and anti angiogenic factors schemes and progresses the physiological angiogenesis. A multitude of biochemical variations and simultaneous destruction of cartilage in OA precipitates synovitis characterized by altered chondrocyte function, enhanced angiogenesis and changes in bone turnover. The sprouting of neo-vessels serves as conduits for the macrophages and lymphocytes to get recruited to the site of inflammation. A plethora of pro inflammatory and pro catabolic mediators released by these macrophages and lymphocytes in turn promote the process of angiogenesis making them inter-dependable variables. The activated M2 macrophages release Pro-angiogenic factors like Vascular endothelial growth factor (VEGF), Tumor necrosis factor -α (TNF-α) and TIMPs which orchesters the process of angiogenesis. On the other hand, the plummeting levels of physiological oxygen tension in the synovial capsule, due to high metabolic demand, causes transcriptional level upregulation of HIF-α (Hypoxia inducible factor –α) which serves as a stimulatory factor for synovial cells and macrophages to produce VEGF promoting angiogenesis. [7]

Arachidonic acid pathway, a crucial mediator of inflammatory cascade is regulated by its key enzyme, prostaglandin endoperoxide synthase, widely known as cyclooxygenase. The type two isoform of this enzyme, COX-2 or PTGS-2 is minimally expressed in quiescent blood vessels and is upregulated during hypoxic conditions. Upon upregulation, it stimulates EGFR/ p38-MAPK /Sp1-Dependent Signalling promoting angiogenesis. It is noted that COX-2 plays a crucial role in the pathogenesis of inflammatory disorders like osteoarthritis [1]. The metabolic products of arachidonic acid pathway like thromboxane A2, PGE2, PGI2 serve as pro-angiogenic mediators [8]. Particularly TXA2 acts a stimulatory factor for capillary tube formation [9,10]. Another crucial AA metabolite, PGE2 activates HIF-α which in turns stimulates the expression of VEGF. Studies have reported that PGE2 activates ERK2/JNK1 pathways by binding to EP2 receptor a crucial mechanism involved in PGE2 mediated VEGF activation [11, 12]. On

the other hand, PGE2 also upregulates the expression of MMP-2 and 9 which are essential in invasion of the leading tip of neo-sprouts [13, 14]. PGI2 is involved in sprouting and maintaining VEGF induced permeability of blood vessels. BCl-2 an anti-apoptotic factor promotes chondrocytes survival but observed to be overexpressed in OA causing induction of angiogenic phenotype by increasing the production of VEGF.

The present study employed Chick Chorioallantoic Membrane (CAM) Assay to study the effect of study molecules on angiogenesis. Results indicate that neovascularisation is significantly decreased in the group of eggs which were treated with the combination of curcumin and diclofenac sodium. Diclofenac sodium acts equipotent on both Cox-1 and Cox-2 whereas curcumin is a potent inhibitor of COX2 and VEGF. Curcumin achieves the inhibition of COX2 and VEGF by blocking IKK pathway which in turn inhibits NF-Kappa B activation. NF-Kappa B being a crucial transcription factor for inflammatory cytokines known to upregulate COX 2 levels in association with p38 MAPK. The additive synergism observed when diclofenac and curcumin are used in combination can be attributed to the efficient inhibition of COX-2 enzyme. Collectively, the combination of these two pharmacological agents can serve as a novel therapeutic strategy in the management of osteoarthritis by potentially targeting angiogenesis

Conflict of interest: Nil.

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